ANGIOTENSIN-(1-7) AS AN ANTINOCICEPTIVE AGENT IN CANCER-INDUCED BONE PAIN

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Abstract

Many cancerous solid tumors, such as breast, metastasize to the bone and induce bone pain (CIBP). CIBP is often severe due to an enhanced inflammation in the bone microenvironment, rapid bone degradation, and disease progression. Opioids are prescribed to manage this pain but may enhance bone loss and induce analgesic tolerance, further compromising patient quality of life. Angiotensin-(1-7) (Ang-(1-7)), a product of Angiotensin II cleavage by Angiotensin-Converting Enzyme 2, binds and activates the Mas receptor, a Gq/11-protein coupled receptor (MasR). Angiotensin-(1-7)/MasR activation modulates the inflammatory signaling after acute tissue insult resulting in altered production of reactive oxygen species and further inflammatory response, yet no studies have investigated whether Ang-(1-7)/MasR play a role in CIBP. Therefore, we hypothesized that Ang-(1-7), acting at the MasR, inhibits CIBP by reducing proinflammatory cytokines/chemokines in an immunocompetent murine model of breast cancer-induced bone pain. Murine breast cancer cells, 66.1, were implanted into the femur of mice and allowed to establish and proliferate for 7 days. Cancer inoculation increased spontaneous and evoked pain behaviors by day 7 that were significantly reduced after a single injection of Ang-(1-7) and after chronic administration (p<0.01); co-administration of a MasR antagonist reversed this reduction. Cytokine/chemokine profiling of bone marrow extrudates from Ang-(1-7) mice of the cancer studies revealed significant increases in the relative expression of C5/C5a, IL-1ra, IL-16, M-CSF, MIG, with concomitant decreases in the expression of MIP-1α compared to vehicle controls (p<0.05). Ang-(1-7) administration normalized levels of IL-1ra and MIP-1α in the bone-tumor microenvironment. Data here suggest that modifying the cancer-induced inflammatory response in the bone-tumor microenvironment with Ang-(1-7) through the Mas receptor significantly attenuates CIBP, which may be an alternative therapeutic strategy for the nearly 70% of advanced stage cancer patients who experience excruciating pain.
Introduction

Bone pain is commonly experienced by 75-90% of late-stage metastatic cancer patients [1]. Metastatic cancer-induced bone pain (CIBP) is the most common type of reported pain but is poorly managed [2]. In 1998, the World Health Organization implemented a three-tiered Pain Relief Ladder for cancer pain relief in adults [3]. This outline suggests that CIBP is initially treated with non-opioids such as non-steroidal anti-inflammatory drugs (NSAIDS), followed by mild opioids, and finally, as the disease progresses, the use of strong opioids such as morphine. Unfortunately, even the strongest of opioids are not enough to provide complete analgesia, while coupled with severe dose-dependent side effects such as nausea, constipation, sedation, impaired cognition, and respiratory depression. Such side effects impede patient quality of life; it is estimated that up to 30% of cancer pain patients do not achieve satisfactory pain relief.

Preclinical modeling of CIBP has revealed many mechanisms driving this complex disease state, leading to the identification of potential therapeutic targets. For instance, CIBP elicits neurochemical changes unlike other chronic pain states [4]. Although the bone is greatly innervated with both sympathetic and nociceptive nerve fibers, many human tumors of the bone are without detectable nerve fibers in the tumor itself or in the adjacent peripheral bone [5, 6]. Contributors to nociceptive signaling associated with CIBP include an acidic tumor environment and the secretion of growth factors, cytokines, and chemokines from the tumor and tumor-associated cells, and enhanced nerve sprouting in the local environment [4, 7-10].

The renin-angiotensin system (RAS), well known for roles in blood pressure regulation and fluid homeostasis, was recently implicated in multiple aspects relevant to metastatic bone disease including inflammation, angiogenesis, tumor cell proliferation, and migration [11, 12]. Angiotensin II (Ang II) is the major end product of the RAS through multiple cleavage steps with angiotensin converting enzyme (ACE). This nonapeptide (i.e., Ang II) binds to and activates two G-protein coupled receptors (GPCRs), angiotensin II receptor type 1 (AT$_1$) and type 2 (AT$_2$) [13]. Physiological effects such as vasoconstriction, inflammation, fibrosis, cellular growth and migration, and fluid retention are reported for AT$_1$ and AT$_2$ [14]. Ang II is cleaved by ACE2 to yield Angiotensin-(1-7), a biologically active heptapeptide. In contrast to Ang II, Ang-(1-7) binds at the $G_{q/11}$–coupled Mas receptor (MasR; $K_d=0.83$ nM) with 60-100 fold greater selectivity for MasR over the AT$_1$ and AT$_2$ receptors [15, 16]. Activation of the MasR elicits effects opposite to those of the Ang II/AT$_1$/AT$_2$ axis: vasodilation, anti-inflammation, anti-fibrosis, and natriuresis [12, 16, 17].
The Mas receptor is expressed throughout the central and peripheral nervous systems in areas linked to nociceptive processing, the dorsal root ganglia (DRG) and spinal cord [18-20]. In a model of prostaglandin-induced hyperalgesia, administration of Ang-(1-7) dose-dependently attenuated peripheral nociceptive effects independent of opioid receptors [18, 20]. Similarly, the co-administration of Ang-(1-7) and Ang II reduced the pronociceptive effects of Ang II in a dose-dependent manner [18]. Ang-(1-7) and Mas expression can significantly reduce pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-6 while also increasing the anti-inflammatory cytokine, IL-10 resulting in the down-regulation of PI3K, phospho-Akt, phospho-p38 MAPK, phospho-JNK, phospho-ERK and phospho-MAPK-2 [21-24]. Although all these factors have been implemented in neuropathic and cancer pain, no reports have investigated the efficacy of Ang-(1-7) in clinically relevant cancer pain models. Given the evidence utility of Ang-(1-7) in inflammatory pain states, we hypothesize Ang-(1-7) decreases CIBP following acute and chronic administration by reducing proinflammatory cytokine release in the bone-tumor microenvironment.
Materials and Methods

In vitro

Cell culture

A murine mammary adenocarcinoma cell line, 66.1, was cultured in Eagle’s minimum essential medium with 10% fetal bovine serum, 100 IU\(^{-1}\) penicillin, and 100 μg/mL streptomycin (P/S). The 66.1 cells were plated in T-75 tissue culture flasks, allowed to grow exponentially, and stored in an incubator at 37°C and 5% CO\(_2\). The cells were counted using a gridded hemacytometer (Hausser Scientific, Horsham, PA, USA) for femoral inoculation.

In vivo

Animals

All procedures were approved by the University of Arizona Animal Care and Use Committee and conform to the Guidelines by the National Institutes of Health and the International Association for the Study of Pain. Female BALB/cAnNHsd mice (Harlan, IN, USA) between 15 and 20g were used in this study. Mice were housed in a climate control room on a 12-hour light/dark cycle and allowed food and water ad libitum. Animals were monitored on days 0, 7, 10, and 14 of the study for clinical signs of rapid weight loss and signs of distress.

Drug treatment

Animals received Angiotensin-(1-7) (Tocris, Ellisville, MO) or the MasR antagonist A-779 (abcam, Cambridge, MA) dissolved in vehicle solutions of 0.9% saline. All intraperitoneal (i.p.) injections were made at a volume of 10 ml/kg. Systemic doses ranged as follows: Ang-(1-7) = 0-100 ug/kg, A-779 = 0-10 ug/kg. A single injection of 0.3 pmol Ang-(1-7) in a 5 μl volume was given for one spinal study. In antagonist studies, A-779 was administered 15 minutes prior to agonist.

Tail Flick

A warm water (52°C) tail flick test was used to determine the effects of Ang-(1-7) during application of a normally noxious stimulus. The distal third of the tails of naïve mice were submerged into the water bath. The withdraw latency, defined as the time for the tail to be withdrawn from the water bath, was recorded. A cutoff time of 10 seconds was enforced to prevent tissue and nerve damage to the tail. Baseline latencies were recorded prior to drug administration. Animals were dosed
systemically with Ang-(1-7) (0-100 μg/kg). Tail flick latencies were reassessed 15, 30, 60, 90, 120, 150, and 180 minutes post-treatment.

**Rotarod**

A rotarod performance test was used to determine the motor and/or sedative effects of Ang-(1-7) (Rotamex 4/8, Columbus Instruments, Columbus, Ohio, USA). Three days prior to testing, naïve mice were subjected to 5 trials in which they were able to acclimate to the rotating rod. On the day of testing, animals were allowed one trial and then baselined. The amount of time the animal remained on the rod was recorded, with a cutoff time of 120 seconds to prevent exhaustion. Animals were dosed (i.p.) as previously described and reevaluated 15, 30, 60, and 120 minutes post-administration.

**Arthrotomy: Intramedullary implantation of 66.1 cells**

For acute and chronic behavioral studies, an arthrotomy was performed, as previously described [4]. Animals were anesthetized with 80 mg/kg ketamine - 12mg/kg xylazine (in a 10 ml/kg volume). The surgical area was shaved and cleaned with 70% ethanol and betadine. The condyles of the right femur were exposed and a hole (0.66 mm) was drilled to create a space for the injection. A 5 μl volume of 66.1 cells (8,000 cells per 1 μl) in MEM (or 5 μl MEM without cells in sham animals) was injected into the intramedullary space of the mouse femora. Proper placement of the injector was confirmed by radiograph (Faxitron X-ray imaging). Holes were sealed with bone cement and the patella reset. Muscle and skin were closed in separate layers with 5-0 vicryl suture and wound autoclips, respectively. Animals were given 0.1 ml of 8mg/ml (10ml/kg volume) gentamicin following surgery to prevent infection. Staples were removed 7 days post-surgery.

**Acute Behavioral Testing**

Fourteen days post-surgery, baseline behaviors of spontaneous flinching and guarding and evoked tactile allodynia were recorded. Flinching was characterized by the lifting and rapid flexing of the inoculated hind paw not associated with walking or movement. Guarding was characterized by the lifting the inoculated hind limb into a fully retracted position under the torso. The total number of flinches and the time spent guarding over the duration of 2 minutes was recorded, and paw withdrawal thresholds were calculated from the animals’ responses to variously weighted filaments. Animals were then separated into treatment groups and dosed systemically with Ang-(1-7) (0-10 μg/kg). Following administration, animals were tested at 15, 30, 60, 90, 120, 150, and 180 minutes post-treatment or until their pain behaviors returned to baseline.
A separate group of animals were dosed with A-779 and Ang-(1-7) in the following manner to assess MasR-dependency: vehicle (0.9% saline at 10 ml/kg) - vehicle injection, vehicle - 0.058 Ang-(1-7), 0.19 μg/kg A-779 - vehicle, or 0.19 μg/kg A-779 - 0.058 μg/kg Ang-(1-7). The second injection was given 15 minutes after the first. Mice pain behaviors were recorded at various time points (15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes post-treatment).

**Chronic Behavioral Testing**

Seven days post-surgery, baseline behaviors of spontaneous pain and tactile allodynia, as described above, were recorded and animals were separated into four groups: 66.1 Ang-(1-7) treated, 66.1 vehicle (0.9% saline) treated, media Ang-(1-7) treated, and media vehicle treated. Animals were then dosed (i.p.) accordingly with either 0.058 μg/kg Ang-(1-7) (at 10 mg/kg) or 10 mg/kg of 0.9% saline. Animals were dosed at the same time each day 7 to 14 days post-surgery. On day 10, prior to dosing, baseline behaviors were recorded, and behaviors were recorded again 15 minutes following treatment. This 15-minute time point is based on the time of peak effect determined by the acute studies. Fourteen days post-surgery, behaviors were again recorded pre- and post-treatment. Animals were sacrificed following treatment and testing on the fourteenth day post-surgery, and the following tissues were collected for biochemical use: serum, femur extrudate, and lumbar dorsal root ganglia.

**Ex Vivo**

**Western Blot Analysis**

Homogenized dorsal root ganglia (DRG) from mice used in behavioral studies were analyzed for expression of MasR. DRGs were homogenized in RIPA buffer and 24 μg of sample was resolved on a 10% SDS–polyacrylamide gels (TGX Criterion XT; Bio-Rad, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (PVDF). Protein transfer was verified by Ponceau S staining, and PVDF membranes were incubated in a blocking buffer of 5% non-fat dry milk in Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST) for one hour at room temperature. Membranes were then incubated with primary antibody: rabbit polyclonal anti-Angiotensin-(1-7) Mas Receptor (Alomone Labs AAR-013; 1:200 dilution) or mouse monoclonal anti-actin AC40 (Cell Signaling 7076S; 1:10,000 dilution) in 1% milk in TBST overnight at 4°C. The membranes were washed in TBST (three quick rinses; three five min washes; three ten min washes) and incubated with appropriate secondary antibodies for 1 hour at room temperature. Membranes were again washed and developed using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ). Bands were quantitated and corrected for background using ImageJ densitometric software (Wayne Rasband, Research Services...
Branch, National Institute of Mental Health, Bethesda, MD). All data were normalized to actin in each lane and reported as fold change over untreated control.

**Cytokine Assay**

Femur extrudates from mice used in behavioral studies were screened for relative levels of forty cytokines. Animals were euthanized on day 14 post-inoculation, as indicated, and the ipsilateral and contralateral femurs were removed. For each femur, the proximal and distal ends were clipped and the intramedullary extrudate was rinsed into a vial; each femur was rinsed six times with phosphate-buffered saline containing protease inhibitor cocktail and EDTA (Pierce, Rockford, IL, USA). Femurs from five animals were pooled per sample. Samples were analyzed with the Proteome Profiler Mouse Cytokine Array Kit (ARY006; R&D Systems, Minneapolis, MN), as outlined by the manufacturer and analyzed with ImageJ densitometric software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). All data were normalized to the reference samples provided by the manufacturer and reported as relative density.

**Statistical analysis**

**Behavioral:** Statistical significance between treatment groups for the dose response curve in acute behavioral studies was determined by one-way ANOVA and Tukey’s t test for unpaired experimental data. Statistical significance between treatment groups for chronic behavioral studies was determined using two-way ANOVA and Tukey’s t test for multiple comparisons. Data are reported as mean ± S.E.M. for n = 5-8 mice/treatment group

**Ex vivo:** Cytokine data are reported as mean ± SD from groups of n = 5.

**General:** A value of p < 0.05 was accepted as statistically significant. Statistical analyses were run and plots generated in GraphPad Prism 5.0 (Graph Pad Inc., San Diego, CA).
Results

Angiotensin-(1-7) increases nociceptive threshold in naïve animals

Previous studies have indicated that Ang-(1-7) has limited antinociceptive efficacy in non-injured animals after peripheral administration [25]. We administered Ang-(1-7) systemically (0.360, 1, 10, and 100 μg/kg, i.p.) in naïve mice and observed a dose-dependent increase in nociceptive threshold as determined by the tail flick test (Fig. 1A). Ang-(1-7) effects peaked 15 mins post administration with 1 μg/kg and lasted for up to 2 hrs (Fig. 2B). Notably, tail flick latencies returned to baseline values at the two highest doses investigated (10 and 100 μg/kg).

To exclude the possibility that Ang-(1-7) administration reduced mobility in order to increase tail withdraw latency, rotorod testing was performed. Naïve animals were trained to walk on a rotating rod for 2 min. After training, mice were injected with Ang-(1-7) by either spinal or systemic routes. No significant differences in rotorod latencies were observed between vehicle and Ang-(1-7) treated mice (results not shown). Together, these data suggest that systemic Ang-(1-7) is antinociceptive after a single administration without noticeable impact on motility.

Acute administration of Angiotensin-(1-7) reduces spontaneous pain behaviors and tactile allodynia through MasR

We next assessed the antinociceptive efficacy of Ang-(1-7) after induction of CIBP. A single bolus of Ang-(1-7) (0.036, 0.360, 1, and 10 μg/kg) or vehicle was injected and pain behaviors were reassessed. Significant reductions in spontaneous pain behaviors onset 15 min after injection and persisted for nearly 2 hours (Fig. 2A, B). Similar observations were made for paw withdrawal threshold, where the time of peak effect was 30 min with a 90 min duration of action (Fig. 2C).

To determine dose-dependence of these effects, we constructed a dose response curve and calculated the area-under the curve. At 15 min, the maximum efficacy of Ang-(1-7) in reducing guarding was determined to be 52.75% and the A₅₀ dose is 0.035 μg/kg; the A₉₀ dose was 0.058 μg/kg (Fig. 2D). The area under the curve showed throughout the duration of the entire time-course, Ang-(1-7) at 0.36 μg/kg and 1 μg/kg produced the most significant change in total nociception with respect to guarding as compared to the vehicle-treated cancer-induced animals (Fig. 2E). Thus, a single injection of Ang-(1-7) is effective against established CIBP.

Next, we investigated the receptor dependence of Ang-(1-7). A-779 (0.19 μg/kg), the selective Mas R antagonist, or saline vehicle was administered 30 minutes prior to Ang-(1-7) (0.058 μg/kg) on day 14 (Fig. 3). Similar to our findings above, Ang-(1-7) reversed CIBP. Inhibition of MasR with A-779
alone did not alter spontaneous or evoked pain thresholds; however, pretreatment with A-779 blocked Ang-(1-7) attenuation of flinching (Fig. 3A), guarding (Fig. 3B), and tactile allodynia (Fig. 3C). These data suggest that Ang-(1-7) elicits antinociception through actions at MasR.

**Chronic administration of Angiotensin-(1-7) attenuates CIBP through MasR**

Sustained administration of many analgesics can lead to the development of tolerance, in which the same dose produces less analgesic efficacy. We next determined if Ang-(1-7) retained antinociceptive activity against CIBP after repeated administration. Ang-(1-7) (0.058 µg/kg, i.p.) was administered daily, beginning 7 days post implantation of 66.1 cells in the femur. Animals were tested for CIBP behaviors on day 7, 10, and 14 both prior to and 15 minutes after treatment. Cancer implantation significantly increased the number of flinches and amount of time spent guarding 7 days post-surgery. Animals experienced significant reduction in guarding (Fig. 4A) and flinching (Fig. 4B) and increase in paw withdraw threshold (Fig. 4C) both prior to and following treatment on days 10 and 14 post-surgery. Vehicle had no significant effect.

Chronicity of pain can alter GCPR dynamics. We next asked if MasR continued to underlie actions of Ang-(1-7) after repeated dosing. A-779 (0.19 µg/kg) was administered 30 minutes prior to Ang-(1-7) (0.058 µg/kg) daily 7-14 days post cancer inoculation (Fig. 5). Administration of A-779 alone had neither a pro- or anti-nociceptive effect on the mice, similar to our observations after a single injection, the chronic co-treatment of A779 with Ang-(1-7) prevented attenuation of CIBP.

**MasR is localized on dorsal root ganglia**

Our findings that Ang-(1-7)/MasR alleviates CIBP suggests that MasR is expressed within pain pathways. We collected lumbar dorsal root ganglion (DRG) from naive, sham, cancer (66.1), and 66.1 Ang-(1-7) treated mice. In naïve BALB/cAnNHsd mice, MasR is expressed in the dorsal root ganglia (Fig. 6); a band was observed at ~50 kDa. Sham surgery (i.e. media only) did not alter MasR expression levels. Introduction of the murine mammary adenocarcinoma line 66.1 into the femoral intramedullary space decreased the expression of MasR in the ipsilateral DRGs at the ~50 kDa molecular weight. Interestingly, a second band at ~40 kDa was detected in these same DRGs (Fig. 6A, lane 3). Ang-(1-7) treatment (0.058 µg/kg, i.p., q.d., 7d) normalized expression of MasR (~50 kDa, Fig. 6B) and increased the relative expression at the ~40 kDa molecular weight marker (Fig. 6C). These data suggest that both cancer in the bone environment and repeated Ang-(1-7) treatment modify MasR expression in the DRG.
Administration of Ang-(1-7) elicits changes in cytokine expression in the femur microenvironment

Inflammation and tumor associated immune cells contribute to CIBP. We next asked how levels of inflammatory mediators are altered during CIBP and if Ang-(1-7) therapy normalized cancer induced changes. Following chronic administration of Ang-(1-7) in the CIBP model, femur extrudate was collected from the contralateral and ipsilateral femurs of all mice. Following a cytokine profile (Fig. 7A), significant changes to a number of cytokines was observed between the cancerous femurs treated with either vehicle or Ang-(1-7). Significant increases in the relative expression of C5/C5a, IL-1ra, IL-16, M-CSF, MIG, and decrease in the expression of MIP-1α compared to vehicle controls in the ipsilateral femurs were observed. The contralateral femurs experienced significant increases in C5/C5a, GM-CSF, CD54, IL-16, IP-10, MIG, TIMP-1, and TNF-α (Fig. 7B). Ang-(1-7) administration normalized expression of IL-1ra and MIP-1α in the ipsilateral cancerous femur (Fig. 7C).
Discussion

Although many primary tumors are not reported to be painful, metastases to the bone cause severe pain. Cancer-induced bone pain is poorly treated by existing medications, such as opioids, despite the implementation of the three-step treatment ladder by the World Health Organization [3]. These guidelines describe a treatment paradigm that advances from non-opioid analgesics through strong opioids with adjuvant supplementation (e.g., bisphosphonates, local radiotherapy) to treat progressively worsening pain [26]. However many of these therapies (i.e. opioids) are associated with severe dose-limiting side effects that further compromise patient quality of life [27]. Here, we demonstrate for the first time Ang-(1-7) at the MasR alleviates cancer-induced bone pain by both acute and sustained systemic administration with limited adverse events. Further, we show that femoral implantation of breast cancer cells shifts MasR expression in the dorsal root ganglia; this shift is sensitive to Ang-(1-7) intervention. Lastly, we found that repeated Ang-(1-7) dosing normalizes the intramedullary content of inflammatory mediators known to contribute to CIBP. Together, our data support the use of Ang-(1-7) as an alternative therapeutic target for CIBP.

The drugs of choice for both acute and chronic pain are NSAIDs and opioids. Opioids have good clinical analgesic efficacy but often require escalating doses to achieve a consistent analgesic efficacy over time [28]. Opioid-associated side effects that limit increased dosing are nausea, constipation, sedation, cognitive deficits, respiratory depression, and an abuse potential [29]. For cancer patients, the more relevant issue is maintaining pain relief. In metastatic bone disease, chronic morphine is associated pre-clinically with enhanced bone loss and increased (2-fold) spontaneous fracture rate, as well as up-regulated IL-1β within the femurs of sarcoma-treated mice [30]. Thus, new, opioid-sparing strategies are needed to ensure these patients are receiving adequate analgesia.

The renin-angiotensin system, widely known for roles in blood pressure regulation and fluid homeostasis, was recently implicated in multiple aspects relevant to metastatic bone disease including inflammation, tumor cell proliferation and migration, and angiogenesis [11, 12]. For this reason, we chose to investigate the utility of Ang-(1-7), a product of Ang II cleavage, in CIBP. Our findings are consistent with those in previous pain-related studies where Ang-(1-7) attenuated PGE₂-induced inflammation [20, 25]. In non-injured mice, Ang-(1-7) showed antinociceptive efficacy. Similarly, acute and chronic systemic administration of Ang-(1-7) significantly reduced the pain behaviors associated with cancer-induced bone pain. Ang-(1-7) induced dose-dependent decreases in spontaneous guarding and flinching and increases in evoked paw withdraw thresholds. Furthermore, repeated administration of Ang-(1-7) attenuated CIBP without signs of tolerance after 7 days. In both treatment paradigms, the antinociceptive actions of Ang-(1-7) were completely reversed by A-779, a Mas
receptor antagonist. While it is possible that Ang-(1-7) may have actions that affect AT₁ expression, we believe these data suggest that activation of the MasR is a valid target for CIBP.

If MasR is a valid therapeutic target for CIBP and the site of action for Ang-(1-7), we would expect changes in MasR expression within the nociceptive circuit. Our data confirms that MasR is expressed in the dorsal root ganglia of naive BALB/c mice, in accordance with a previous model of inflammatory pain [20]. However, our band for MasR emerged at ~50 kDa, while others show MasR at 37 kDa in the retina [31], at 49 kDa in the skeletal muscle [32], and at 83 kDa in HEK293T cells [33]. Such inconsistency in molecular weight is for the most part unexplained; however, there are three different sites of glycosylation on MasR, which may help to explain the differences in molecular weight, although the impact of such glycosylation is unknown. In media inoculated sham mice, the relative density of MasR to β-actin was unchanged compared to naïve controls, suggesting that injury in the form of surgery did not alter MasR expression. In contrast, implantation of breast cancer cells reduced relative expression of ~50 kDa band. Furthermore, cancer induced the emergence of a second band at ~40 kDa suggesting that MasR may be modified in this pathological state. Interestingly, both the ~50 and ~40 kDa MasR in the dorsal root ganglia were increased with daily Ang-(1-7) administration; the ~50 kDa band was normalized in Ang-(1-7) treated cancer animals. Thus, the presence of the MasR in the DRGs in our model and subsequent expression changes with cancer inoculation and Ang-(1-7) administration supports the role of Ang-(1-7) as a potential therapeutic target for CIBP.

Increases in pro-inflammatory mediators are associated with advanced stages of cancer and are known to play a role in CIBP [4]. We found that implantation of 66.1 cells increases the relative expression of C5/C5a, IL-1ra, IL-16, M-CSF, MIG, and decreases the expression of MIP-1α compared to vehicle controls within the intramedullary space. The contralateral femurs experienced significant increases in C5/C5a, GM-CSF, CD54, IL-16, IP-10, MIG, TIMP-1, and TNF-α. Of these, daily Ang-(1-7) administration normalizes the expression of IL-1ra and MIP-1α in the bone-tumor microenvironment. IL-1ra is antagonist for the IL-1 soluble receptor. Such increase in IL-1ra may lead to a decrease in the expression of immunocompetent cells, such as phagocytes and lymphocytes, and the overall decrease in inflammatory response. On the other hand, cancer inoculation increases the levels of MIP-1α, which are in tum decreased with Ang-(1-7) administration. A decrease in MIP-1α may lead to the decrease in recruitment and activation of leukocytes. Together, the overall decrease in the expression, recruitment, and activation of immunocompetent cells may lead to a decrease in the activation of the inflammatory immune response, thereby reducing pain. In terms of transcription, the beta and gamma subunits of the G_{q/11} protein associated with MasR can activate the MAPK pathway, leading to transcription of both of these cytokines. Therefore the binding of Ang-(1-7) to MasR plays an important role in the
transcription of cytokines and the resulting recruitment of the immune system. Thus, Ang-(1-7) combats the inflammatory response resulting from tumor metastasis within the bone-tumor microenvironment, serving as a potential mechanism for the results presented in this study.

The exact mechanisms by which Ang-(1-7) exerts its antinociceptive effect are unknown. In addition to regulating transcription of cytokines, Ang-(1-7)/MasR activation of the MAPK pathway can decrease transcription of norepinephrine transporters, leading to an increase of norepinephrine in the synaptic cleft. Thus, norepinephrine will serve as a neurotransmitter to increase 2nd order inhibitory neuron signaling to inhibit pain signaling. Moreover, Ang-(1-7) binding to MasR may inhibit phosphorylation of p38 MAPK [18]. Phosphorylation of spinal p38 MAPK has been observed in chronic injury [34], and therefore a decrease in phosphorylation of p38 MAPK in chronic injury models such as cancer-induced bone pain may prove to yield antinociceptive effect. MasR activation by Ang-(1-7) can activate nitric oxide synthase, increasing intracellular NO [25] to further increase intracellular levels of cyclic GMP (cGMP). Cyclic GMP production leads to the activation of ATP-dependent potassium channels, thereby hyperpolarizing the neuron [35-37], in addition to blocking voltage-gated calcium channels which will slow or block neurotransmitter release. Together, the actions of Ang-(1-7)/MasR through cGMP can lead to a decrease in nociception through a decrease in neuronal firing of nociceptive fibers. Lastly, the Mas receptor can dimerize with the AT1 receptor [33, 38] where it acts as an antagonist to the receptor. In doing so, such dimerization can prevent Ang II from binding, inhibiting the pronociceptive effects of the peptide. Further research is warranted to elucidate the mechanism by which the Ang-(1-7)/MasR axis is acting to reduce CIBP.

Our results thereby confirm the utility of further investigating GPCRs as targets for cancer therapeutics. Recently, it has been discussed that the GPCRs of the renin-angiotensin system play a significant role in breast cancer [39], and most specifically the Ang-(1-7)/MasR axis, as it has been shown to be protective against cancer. Stimulation of MasR via Ang-(1-7) treatment has been shown to have antiproliferative effects against tumor growth [40-42] without major effects. Additionally, Ang-(1-7) treatment in a metastatic prostate cancer model has been shown to lead to a reduction of osteoclastogenesis [43], suggesting Ang-(1-7) prevents the formation of osteolytic lesions. Such reduction of osteoclastogenesis may reduce tumor survival in the bone-tumor microenvironment, further serving in part as an explanation of the reduction in CIBP due to Ang-(1-7) administration shown in our study. Thus, the use of Ang-(1-7) in treating cancer-induced bone pain is a highly favorable and safe alternative or adjuvant therapeutic to the current treatment with strong opioids such as morphine.
Figures

Figure 1

A

**Ang-(1-7) Tail Flick**

- ○ Vehicle (Saline)
- □ Ang-(1-7) 0.360 µg/kg
- ▲ Ang-(1-7) 1 µg/kg
- ▼ Ang-(1-7) 10 µg/kg
- ◆ Ang-(1-7) 100 µg/kg

B

**Tail Flick Dose Response Curve**

TF%Antinociception (± SEM)

TF Latency (s ± SEM)

Time (min)

Baseline 15 30 60 90 120 150 180

Ang-(1-7) (µg/kg), 15 min

- ***
- *
- #
Figure 3

A  A-779 and Ang-(1-7) Guarding

- Saline, Saline
- Saline, Ang-(1-7) 0.058 µg/kg
- A-779 0.19 µg/kg, Saline
- A-779 0.19 µg/kg, Ang-(1-7) 0.058 µg/kg

B  A-779 and Ang-(1-7) Flinching

- Saline, Saline
- Saline, Ang-(1-7) 0.058 µg/kg
- A-779 0.19 µg/kg, Saline
- A-779 0.19 µg/kg, Ang-(1-7) 0.058 µg/kg

C  A-779 and Ang-(1-7) Von Frey

- Saline, Saline
- Saline, Ang-(1-7) 0.058 µg/kg
- A-779 0.19 µg/kg, Saline
- A-779 0.19 µg/kg, Ang-(1-7) 0.058 µg/kg
Figure 4

A. Guarding

B. Flinching

C. von Frey

Legend:
- Sham, Saline
- Sham, 0.058 µg/kg Ang-(1-7)
- 66.1, Saline
- 66.1, 0.058 µg/kg Ang-(1-7)
Figure 6

(A) Naive Sham Saline Ang-(1-7)

(B) Fold Change Over Naive Normalized to β-actin

Mas Receptor (~50kDa)

0.0 0.5 1.0 1.5

Naive Sham 66.1 66.1 + Ang-(1-7)

(C) Fold Change Over Naive Normalized to β-actin

Mas Receptor (~40kDa)

0 5 10 15

Naive Sham 66.1 66.1 + Ang-(1-7)
### Figure 7

#### A

**66.1 Saline Ipsilateral**

**66.1 Ang-(1-7) Ipsilateral**

#### B

<table>
<thead>
<tr>
<th>Cytokine/Chemokine</th>
<th>66.1 mammary adenocarcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline Ipsilateral vs. Contralateral</td>
</tr>
<tr>
<td>C5/C5a</td>
<td>increased</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>increased</td>
</tr>
<tr>
<td>sICAM-1 (CD54)</td>
<td>increased</td>
</tr>
<tr>
<td>IL-1ra (IL-1F3)</td>
<td>decreased</td>
</tr>
<tr>
<td>IL-16</td>
<td>increased</td>
</tr>
<tr>
<td>IL-23</td>
<td>increased</td>
</tr>
<tr>
<td>IP-10 (CXCL 10)</td>
<td>increased</td>
</tr>
<tr>
<td>KC (CXCL1)</td>
<td>increased</td>
</tr>
<tr>
<td>M-CSF</td>
<td>increased</td>
</tr>
<tr>
<td>JE (CCL2/MCP-1)</td>
<td>increased</td>
</tr>
<tr>
<td>MCP-5 (CCL12)</td>
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</tr>
<tr>
<td>MIG (CXCL9)</td>
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</tr>
<tr>
<td>MIP-1a (CCL3)</td>
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</tr>
<tr>
<td>MIP-2 (CXCL2)</td>
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<tr>
<td>RANTES (CCL5)</td>
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</tr>
<tr>
<td>SDF-1 (CXCL12)</td>
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</tr>
<tr>
<td>TIMP-1</td>
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<tr>
<td>TNF-a</td>
<td>increased</td>
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</tbody>
</table>

#### C

- **IL-1ra**
  - 66.1 Saline Contralateral
  - 66.1 Ang-(1-7) Contralateral
  - 66.1 Saline Ipsilateral
  - 66.1 Ang-(1-7) Ipsilateral

- **MIP-1 α**
  - 66.1 Saline Contralateral
  - 66.1 Ang-(1-7) Contralateral
  - 66.1 Saline Ipsilateral
  - 66.1 Ang-(1-7) Ipsilateral

**Relative Density (± SD)**

- **IL-1ra**: ns
- **MIP-1 α**: **ns**
Table and Figure Legends

**Figure 1**
Effect of one-time administration of Ang-(1-7) in naïve mice. Tail flick latencies were recorded before administration (baseline) and at various time points after drug administration. **(A)** Time of peak effect at 15 minutes post-administration lasting for two hours. **(B)** Dose of 1 μg/kg most effective in increasing nociceptive threshold. Values represent the means ± standard error of the mean, n=5-8, *p< 0.05 and ***p<0.001 compared to vehicle and #p<0.05 1 μg/kg compared to 100 μg/kg.

**Figure 2**
Effect of one-time administration of Ang-(1-7) 14 days post-inoculation. Spontaneous pain behaviors (A) guarding and (B) flinching were recorded in a two minute period, and evoked (C) von Frey paw withdrawal thresholds were measured pre-surgery, post-surgery (14 days post inoculation), and at various time points after drug administration. Time of peak effect at 15 minutes post-administration lasting for two hours. **(D)** E$_{max}$= 52.75%, A$_{50}$= 0.035 μg/kg, A$_{90}$= 0.058 μg/kg for the guarding curve. **(E)** Area under the curve demonstrates the dose-dependency of the actions of Ang-(1-7). Values represent the means ± standard error of the mean, n=5-8, **p<0.01 and ***p<0.001 compared to vehicle.

**Figure 3**
Effect acute coadministration of Ang-(1-7) (0.058 μg/kg) and A-779 (0.19 μg/kg) 14 days post-inoculation. Animals were dosed with A-779 30 minutes prior to administration of Ang-(1-7). Spontaneous pain behaviors (A) guarding and (B) flinching were recorded in a two minute period, and evoked (C) von Frey paw withdrawal thresholds were measured pre-surgery, post-surgery (14 days post inoculation), and at various time points after drug administrations. Administration of A-779 completely reversed the effects of Ang-(1-7) in the CIBP model. Values represent the means ± standard error of the mean, n=5.

**Figure 4**
Chronic pain behaviors reduced with daily administration of Ang-(1-7) (0.058 μg/kg) 7-14 days post-inoculation. Spontaneous (A) guarding and (B) flinching, as well as evoked (C) von Frey paw withdraw thresholds were recorded pre-surgery, post-surgery (7 days post inoculation), and both
before and after drug administration on days 10 and 14. Values represent the means ± standard error of the mean, n=5, *p<0.05, ***p<0.0001.

**Figure 5**
Effect chronic coadministration of Ang-(1-7) (0.058 μg/kg) and A-779 (0.19 μg/kg). Animals were dosed days 7-14 post-surgery with A-779 30 minutes prior to administration of Ang-(1-7). Spontaneous pain behaviors (A) guarding and (B) flinching were recorded in a two minute period, and evoked (C) von Frey paw withdrawal thresholds were recorded pre-surgery, post-surgery (7 days post inoculation), and both before and after drug administration on days 10 and 14. Daily administration of A-779 completely reversed the effects of Ang-(1-7) in the CIBP model. Values represent the means ± standard error of the mean, n=5, *p<0.05, **p<0.01 and ***p<0.001 compared to 66.1 – Saline, Ang-(1-7).

**Figure 6**
(A) Ipsilateral DRGs were harvested from naïve, sham (media inoculated) or chronically-treated 66.1 inoculated mice, homogenized, and probed for MasR expression via Western blotting. 66.1 inoculation decreased ~50 kDa MasR expression (B) and induced expression of a ~40 kDa band (C). Ang-(1-7) (black bars, B,C) normalized MasR expression in the DRG at ~50 kDa, but increased expression ~40 kDa band. B-actin serves as the loading control. (n=1-2 pools of 5 animals)

**Figure 7**
(A) Representative dot blot images of the 40 cytokine/chemokine panel from femur extrudates. (B) Cancer significantly increased C5/C5a, sICAM-1, IL-16, IP-10, M-CSF, MCP-1, MCP-5, MIP-1α, and TIMP-1 (red), and decreased IL-1ra and RANTES (blue) in the ipsilateral femur as compared to the contralateral femur. Ang-(1-7) significantly increased the relative expression of C5/C5a, IL-1ra, IL-16, M-CSF, MIG, and decreased the expression of MIP-1α compared to vehicle controls. (C) Ang-(1-7) normalized the relative expression of IL-1ra and MIP-1α in the ipsilateral femur compared to contralateral femur.
References


